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Five Intermediate Complexes in Transcription Initiation by RNA Polymerase II

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Summary

A native gel electrophoresis DNA binding assay was used to resolve complexes formed on the adenovirus Major Late Promoter by general transcription factors and RNA polymerase II. Five sets of complexes containing distinct components were identified. These complexes were generated by sequential binding of TFIID, TFIIA, TFIIB, RNA polymerase II, and TFIIE. The relative positions of each of the factors in the complexes were determined by DNAase I footprint analysis. TFIIA, derived from yeast or mammalian cells, formed a complex with yeast TFIID and the TATA element. TFIIB bound to this complex and probably acts as a "bridge" to the polymerase and the initiation site. The addition of ATP or dATP, necessary for "activation" of transcription, resulted in an alteration of the footprint in the +20 to +30 region, the same area protected upon addition of TFIIE to the initiation complex. Addition of ribonucleotide triphosphates generated new complexes that contained accurately initiated transcripts associated with the transcription machinery and the template DNA. A model for the interactions of components in initiation of transcription by RNA polymerase II is proposed.

Introduction

The rate of of initiation of transcription is determined by factors that recognize sequences in enhancer elements, sites upstream of the TATA element, and the TATA element. The upstream binding factors probably influence events at the TATA element; promoting either formation of a complex or initiation by the polymerase. Analysis of the regulation of transcription has been limited by a lack of understanding of the TATA-directed initiation process [illegible]

[illegible] 1987; Parente 1988, for review). Some aspects of the basic initiation reaction are also conserved, as a TATA binding protein from yeast will direct initiation by mammalian factors and polymerase (Buratowski et al., 1988; Cavallini et al., 1988). This conservation in mechanism is also reflected in the conserved structure of RNA polymerase II (pol II) over evolution (Allison et al., 1985; Corden et al., 1985).

Purified pol II will not accurately initiate transcription in vitro. Accurate initiation can be observed in whole cell (Weil et al., 1979; Manley et al., 1980) or nuclear extracts (Dignam et al., 1983a), and can be reconstituted with partially purified fractions. In addition to pol II, four activities have been shown to be required. These general transcription factors have been partially purified and designated TFIIA, -B, -D, and -E (Matsui et al., 1980; Samuels et al., 1982; Dignam et al., 1983b; Sawadogo and Roeder, 1985a). This combination of transcription factors will accurately initiate from a minimal promoter containing only a TATA element and start site.

The factor TFIID (also known as DB or BTF1) contains a protein that specifically binds to the TATA element (Sawadogo and Roeder, 1985b; Nakajima et al., 1988). The first step in the initiation of transcription is correlated with this binding (Davison et al., 1983; Fire et al., 1984). Recently, it has been shown that TFIID from the yeast Saccharomyces cerevisiae is functionally interchangeable in vitro with mammalian TFIID (Buratowski et al., 1988; Cavallini et al., 1988). The two proteins may differ in some physical characteristics, as the purified yeast TFIID behaves as a single protein of approximately 25 kilodaltons (Buratowski et al., 1988), while the mammalian factor behaves as a much large entity (Samuels et al., 1982; Reinberg et al., 1987).

The factor TFIIA (AB, STF) is also required for the efficient interaction of TFIID and the TATA element (Davison et al., 1983; Fire et al., 1984), although its mechanism of action is unknown. TFIIA activity has been purified from calf thymus and HeLa cells as a set of proteins of 19 and 13 kd (Samuels and Sharp, 1986) and as a single protein of 43 kd (Egly et al., 1984), respectively. TFIIA may exist as a dimer, or these proteins may represent proteolytic products, as native size estimates of cruder preparations suggest a molecular weight of 34 kd for TFIIA from calf thymus (Samuels and Sharp, 1986) and 82 kd for TFIIA from HeLa cells (Reinberg et al., 1987).

The factor TFIIB (CBI, BTF3) has been purified from HeLa cells as a protein of 27–30 kd (Reinberg and Roeder, 1987a; Zheng et al., 1987). Although its specific function is unknown, it binds to pol II and possibly TFIIE in solution [illegible] (Reinberg and Roeder, 1987a). The only hint to its function in transcription is that fractions containing partially purified TFIIE also contain a DNA-dependent ATPase (Sawadogo and Roeder, 1984; Reinberg and Roeder, 1987a). Gel filtration and sedimentation analysis suggest a molecular weight of 76 kd for the TFIIE activity (Reinberg and Roeder, 1987a). TFIIE is probably related to the

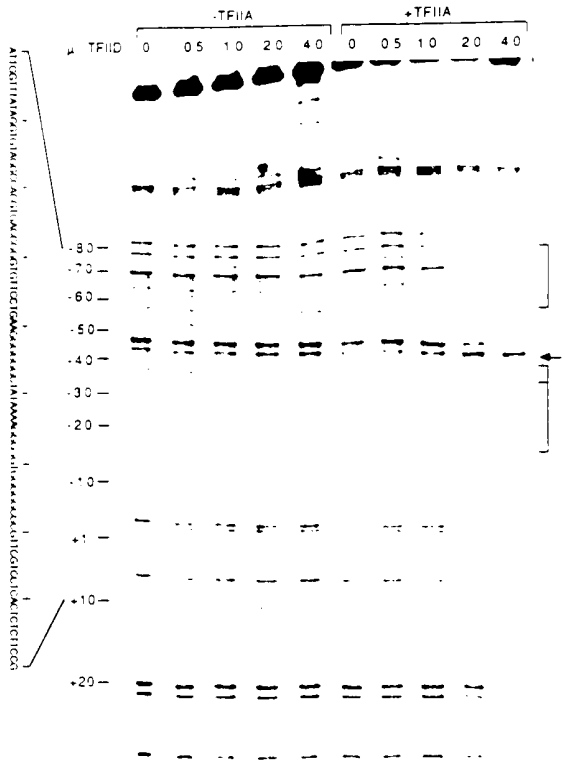

Figure 1. Effect of TFIIA on the DNAase I Footprint of TFIID on the MLP

The reactions contained the indicated amounts of yeast TFIID (Superose-12 fraction) with (+TFIIA) or without (–TFIIA) 2 μl of purified calf thymus TFIIA. The probe contained MLP sequences from –170 to +33 relative to the transcription initiation site (indicated by the numbers), and was labeled on the coding strand. Binding reactions, DNAase I digestions, processing, and electrophoresis were performed as described in Experimental Procedures. The protected regions are bracketed and the arrow points out a site that exhibits a TFIIA-dependent three-fold enhancement in cleavage (as determined by densitometry and normalization to other sites removed from the footprint). The MLP TATA element is located at positions –30 to –25. The sequence TTTATA (TATAAA on the opposite strand) occurs at positions –75 to –70.

RAP (RNA polymerase associated proteins) 30/74 complex, which was originally identified and subsequently purified by affinity chromatography over a column containing RNA pol II (Sopta et al., 1985). RAP 30/74 is required for transcription initiation and may also contain a DNA-dependent ATPase activity (Burton et al., 1986; Burton et al., 1988). Fractions containing partially purified TFIIE can restore transcription to extracts depleted of RAP 30/74

[illegible] complexes of general factors, polymerase and template have been analyzed by low resolution methods such as resistance to challenges by inhibitors and template commitment assays, as well as the higher resolution assay of DNAase I footprinting. To date, initiation complexes have not been resolved by native gel electrophoresis, a method that combines high resolution and sensitivity. Using this DNA binding assay, and either purified or partially purified general transcription factors, we demonstrate here a hierarchy of specific protein–promoter DNA complexes. Each complex requires a specific subset of transcription factors for formation and shows a distinctive DNAase I protection pattern. The complexes react to inhibitor challenges and nucleotide triphosphates in a manner consistent with previous studies of transcription initiation complexes. The hierarchical nature of the complexes suggests a model for transcription initiation and predicts functions for the general factors.

Results

TFIIA Affects Binding of TFIID to the TATA Element

RNA pol II transcription can be inhibited by poly(dI·dC) or Sarkosyl. Preincubation of the transcription template with TFIID and TFIIA before addition of inhibitor renders transcription resistant to such a challenge (Fire et al., 1984; Reinberg et al., 1987). This and other assays have been used to define the first stable complex in initiation: a preinitiation complex "committed" to transcription of a particular template, presumably involving specific binding of TFIID to the TATA element. Although it clearly acts at this step, there has been a variable requirement for TFIIA. At high levels of yeast TFIID, TFIIA was not required for poly(dI·dC)-resistant complex formation, but at lower TFIID levels, TFIIA significantly increased complex formation (Buratowski et al., 1988). This suggests that TFIIA stimulates binding of TFIID to the TATA element. To examine this issue further, increasing amounts of yeast TFIID were tested for DNAase I protection of the adenovirus Major Late Promoter (MLP) in the presence or absence of TFIIA. The TFIID was purified to near homogeneity from yeast whole cell extracts (Buratowski et al., 1988). TFIIA (the generous gift of M. Samuels) was purified to near homogeneity from calf thymus (Samuels and Sharp, 1986). Both factor preparations were purified on the basis of their ability to substitute for the corresponding HeLa factor, and will efficiently function together in an in vitro transcription reaction containing pol II from calf thymus and TFIIB and TFIIE from HeLa cells.

The presence of TFIIA, which demonstrated no protection by itself, induced two changes in the DNAase I footprint of TFIID (Figure 1). The first change was quantitative: approximately two-fold less TFIID was required to saturate binding of the MLP TATA element in the presence of TFIIA. Furthermore, another TATA sequence (TATAAA), at –70 to –75 on the opposite strand, bound TFIID only in the presence of TFIIA, even at the highest levels of TFIID tested

[illegible] Chodosh et al., 1988a). The second change seen was qualitative. In the absence of TFIIA, TFIID protected the coding strand from –17 to –39. In the presence of TFIIA, the protection was extended to approximately –42, and the frequency of cleavage at the upstream boundary increased three-fold (denoted by an arrow in Figure 1). This enhanced cleavage is not due to interactions be-

Figure 2. TFIIA and TFIID Are Required for Specific and Stable Complex Formation on the MLP

(A) Gel shift analysis of complexes formed by TFIID and TFIIA on the MLP. Probe DNA containing MLP sequences from −53 to +33 (labeled on the coding strand) was incubated with 2 μl of purified calf thymus TFIIA (lane 1), 2 μl of purified yeast TFIID (lane 2), or both (lane 3). Binding reactions and electrophoresis of complexes were performed as described in Experimental Procedures. TFIIA formed no complexes, and TFIID formed one complex (NS) that appeared to be nonspecific (see part B and text). Combination of TFIIA and TFIID caused the formation of a new complex (A+D) that was specific for the TATA element.

(B) DNAase I patterns of DNA in the complexes. Binding reaction (scaled up 5-fold) was performed as in (A), lane 3. The reaction was briefly treated with DNAase I before loading of the native gel. After electrophoresis, DNA from each of the complexes was recovered, processed, and electrophoresed on a denaturing gel as described in Experimental Procedures. Free DNA (F, lane 3) and DNA in the nonspecific complex (NS, lane 4) showed no protection, while DNA in the complex dependent on both TFIIA and TFIID (A+D, lane 5) was protected over the TATA element (indicated by bracket). A site of enhanced cleavage (previously observed in solution studies only when TFIIA and TFIID were present) is indicated by an arrow. Numbers indicate position relative to transcription initiation site (+1). G+A and G (lanes 1 and 2) are sequencing ladders of the probe fragment.

tween the two TATA boxes, as it was also observed with a
Figu
TFIIA
TFIID DNAase I footprint (data not shown). Therefore, TFIIA apparently alters and increases the binding of TFIID to the TATA element.

TFIIA Complexes with TFIID and the TATA Element

Several mechanisms can be postulated for the effect of TFIIA on TFIID binding. TFIIA could modify TFIID and/or the promoter DNA to allow a higher affinity interaction. Alternatively, TFIIA could be a component of the preinitiation complex. To explore these possibilities, the gel shift assay was tested for resolution of protein–DNA complexes containing TFIID (Figure 2A). Incubation of TFIIA alone did not yield a complex with the MLP (lane 1), in agreement with previous studies (Egly et al., 1984; Samuels and Sharp, 1986; Reinberg et al., 1987). Addition of TFIID alone yielded a fast migrating protein–DNA complex (lane 2). However, this complex was due to nonspecific binding of a protein, as it was competed by addition of unrelated fragments and formed on fragments not containing a TATA sequence (data not shown). It is thought that this complex is due to a contaminating DNA binding protein, as it does not chromatograph exactly with the TFIID transcription activity (data not shown). When TFIID and TFIIA were incubated together with the MLP probe DNA, a new set of complexes was formed (lane 3). These complexes did not form on a probe from a fragment containing a double point-mutation (TAGAGAA) in the TATA element (data not shown). For footprint analysis, the binding mixture was treated with DNAase I before loading on the native gel, and DNA in the complexes was recovered (Figure 2B). The free probe (F) and the nonspecific complex (NS) yielded the same DNAase I pattern as DNA in solution (lanes 3 and 4). The specific complexes, dependent on TFIIA and TFIID, showed protection of the TATA element (lane 5). Furthermore, this footprint had the 5′ extension and the enhanced cleavage at the upstream site observed previously only when both TFIID and TFIIA were added to a footprint assay in solution. Since the specific complexes always behaved identically and probably resulted from heterogeneity in the purified TFIIA (see below), they will be referred to as a single complex.

The gel shift assay suggested that TFIIA was required to form a stable and specific complex between TFIID and the TATA element, but it did not address whether TFIIA was part of the complex. Knowing that different sources of TFIIA yield factors of different apparent molecular weights (Egly et al., 1984; Samuels and Sharp, 1986; Reinberg et al., 1987), various samples of TFIIA were tested with and without TFIID in the gel shift assay (Figure 3). HeLa fraction [AB] (a second column preparation of human TFIIA; Samuels et al., 1982) and a TFIIA-like activity partially purified from yeast (unpublished data) both failed to form specific complexes in the absence of TFIID (lanes 3 and 4, respectively). However, in the presence of TFIID, a single new complex was formed in each case (lanes 6 and 7). These complexes showed the same DNAase I protection and specificity as the calf thymus TFIIA-dependent

interchangeable in transcription reaction in vitro and produce the same changes in the TFIID footprint, the different mobilities of their complexes with TFIID and the MLP suggest that TFIIA is a component of the complex. The different properties of TFIIA from various sources might reflect inherent differences between species and/or partial proteolytic cleavages

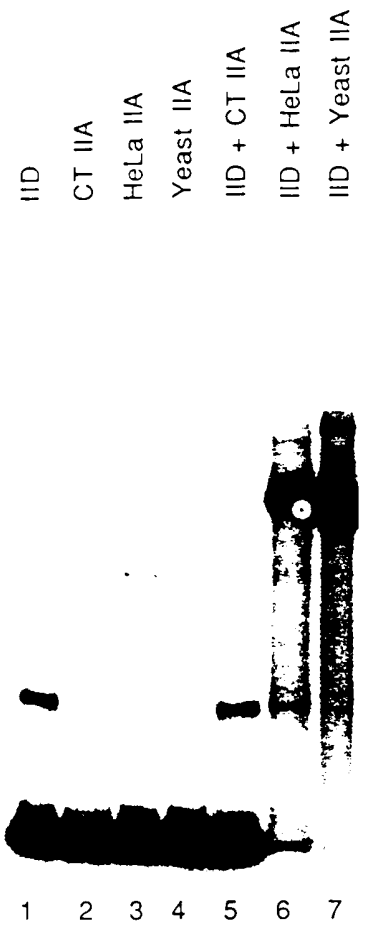

Figure 3. Different Sources of TFIIA Generate Different Mobility Complexes with TFIID on the MLP, Suggesting that TFIIA Is a Component of the Complex

Probe (from plasmid pRW, containing MLP sequences from −53 to +33) was incubated with 2 μl of yeast TFIID (lane 1), 2 μl of TFIIA purified from calf thymus (CT IIA, lane 2), 0.5 μl of HeLa fraction [AB], a partially purified preparation of human TFIIA (lane 3), or 1.0 μl of a partially purified yeast activity that substitutes for TFIIA (Yeast IIA, lane 4). No specific complexes were formed by any of the factors alone. Each of the TFIIA sources was then tested in the presence of 2 μl of TFIID (lanes 5–7). In each case, a new complex was formed. Although the three complexes were of different mobilities, all three showed the same DNAase I protection pattern over the TATA element as described in Figure 2. The TFIIA+TFIID complex in lane 5 is more clearly visible upon darker exposure, and is identical to that shown in Figure 2A, lane 3. A lighter exposure of the gel is shown here so that the different positions of the complexes in lanes 6 and 7 can be distinguished.

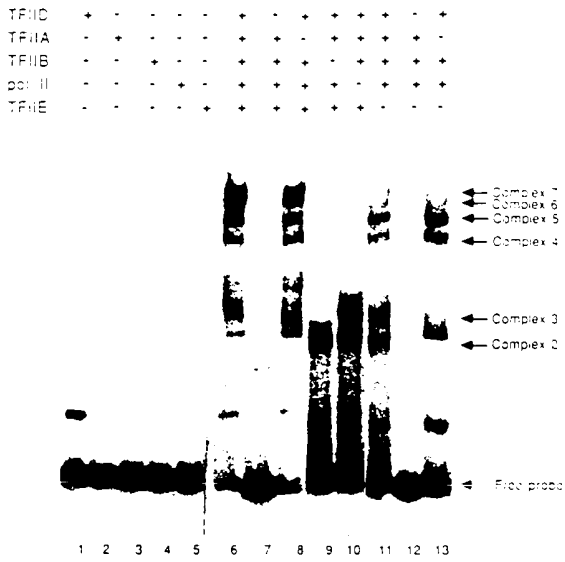

Figure 4. A Hierarchy of Specific Complexes Generated by the General Transcription Factors and Pol II on the MLP

Yeast TFIID (2.0 μl), 2.0 μl of calf thymus TFIIA, 0.3 μl of calf thymus pol II, 0.5 μl of HeLa fraction [CBA] (TFIIB), and 1.0 μl of HeLa fraction [CBB] (TFIIE) were incubated with MLP probe (−53 to +33 plus polylinker sequences) in the indicated combinations. Binding reactions and native gel electrophoresis were carried out as described in Experimental Procedures.

Higher Order Complexes

Identification of a specific complex containing TFIIA and

[illegible] This was tested by incubating the MLP probe with p[illegible] rified TFIID, TFIIA, and pol II, as well as partially purified TFIIB and -E (Figure 4). Individually (lanes 1–5), none of these fractions yielded complexes except for the non[illegible] specific complex formed in the TFIID reaction (lane [illegible]). When all the fractions were incubated together (lane [illegible]) although a complex migrating close to complexes 2 and 3 was observed. This can be seen more clearly by comparing lanes 11 and 13, where less probe is shifted into higher order complexes, and there is less background due to the absence of nonspecific binding proteins contained in the TFIIE fraction. There are several possible ex[illegible]

in addition to the TFIIA+TFIID complex (complex 2), five other complexes were formed (complexes 3–7). These complexes were shown to be specific in two ways. First, each was competed 3- to 5-fold more efficiently by a fragment containing MLP sequences spanning the TATA element (−42 to −17) than by an oligonucleotide that was identical, except for the mutations TATAAAA→TAGAGAA (data not shown). These same two fragments compete with similar relative efficiencies for the DNAase I protection footprint of TFIID on the MLP TATA element (Buratowski et al., 1988). Second, labeled probes of the aforementioned fragments were tested in the gel shift assay. Complexes 2–7 formed on the wild-type probe, but not on the mutant probe (data not shown). This suggests that these complexes are specific for a TATA sequence, and that the only sequences required for their formation reside in or near the TATA element.

Individual fractions were omitted from the binding mixture to test the effect on complex formation. In the absence of TFIID (Figure 4, lane 7), none of the specific complexes were formed. A background of nonspecific complexes was obtained that was due to contaminating

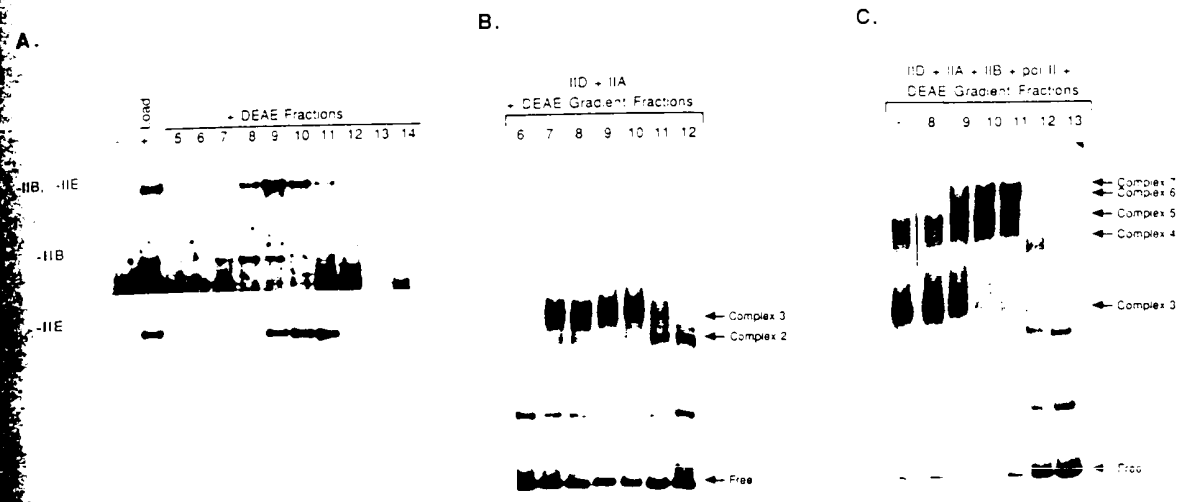

Figure 5. Separation of TFIIB and TFIIE by Gradient Elution Chromatography over DEAE-Sephacel

(A) Transcription assay of gradient fractions for TFIIB and TFIIE activity. Assays were performed as described in Experimental Procedures. Reactions received the following additions: none (−), 0.5 μl of HeLa fraction [CB] (+Load), or 1.0 μl of each of the DEAE-Sephacel gradient fractions. Fractions were tested for the presence of TFIIB and TFIIE together (−IIB, −IIE), or for TFIIB alone (−IIB) or TFIIE alone (−IIE).

(B) Gel shift analysis of DEAE-Sephacel gradient fractions in the presence of TFIIA and TFIID. All reactions contained 2.0 μl of yeast TFIID and 2.0 μl of calf thymus TFIIA and probe containing MLP sequences from −53 to +33. One microliter of each of the gradient fractions was added and incubation and native gel electrophoresis were carried out as described in Experimental Procedures.

(C) Gel Shift analysis of DEAE-Sephacel gradient fractions in the presence of TFIID, TFIIA, TFIIB, and pol II. All reactions contained 2.0 μl of yeast TFIID, 2.0 μl of calf thymus TFIIA, 0.5 μl of calf thymus pol II, and 1.0 μl of DEAE-Sephacel gradient fraction 7 as a source of TFIIB. Reactions received either no addition (−) or 1.0 μl of each of the DEAE-Sephacel gradient fractions.

planations for complex formation in the absence of added TFIIA. The simplest is the possibility that the TFIIB fractions were contaminated with TFIIA activity. Alternatively, it is possible that TFIID and TFIIB, and subsequently the other factors, can form stable complexes in the absence of TFIIA. Potentially, formation of these complexes could be stimulated by TFIIA in the transcription reaction. In either case, these results are consistent with previous experiments showing that an in vitro transcription reaction using these fractions is only partially dependent upon addition of TFIIA (Buratowski et al., 1988). Leaving out the crude fraction containing TFIIB allowed formation of complex 2, but not complexes 3–7 (lane 9). Omission of purified pol II resulted in formation of complexes 2 and 3, but not 4–7 (lane 10). All complexes except 6 and 7 were obtained when the fraction containing TFIIE was not added (lane 11). The omission of TFIID or TFIIA in combination with the absence of TFIIE (lanes 12 and 13, respectively) had the same effects on complex formation as when TFIIE wa

tiall

and pol II used in the previous analysis, further confirmation that formation of the complexes was dependent on the actual transcription factors, and not some other protein in those fractions, was sought. For this, formation of complexes was tested after more extensive fractionation of the transcription activities. Fraction [CB], containing both TFIIB and TFIIE, was chromatographed by salt gradient elution over a DEAE-Sephacel column. The transcription activities of TFIIB and TFIIE were assayed for either together (−TFIIB, −TFIIE) or separately (Figure 5A). TFIIB activity was found predominantly in fractions 7–10, peaking in fraction 8 (−TFIIB). There is also a small amount of TFIIB activity in fraction 11, as seen in the −TFIIB, −TFIIE reactions. TFIIE activity was found in fractions 9–11 (−TFIIE). The same fractions were assayed in the gel shift system by mixing with TFIIA and TFIID (Figure 5B). Complex 3 formed upon addition of fractions 7–10, with some in fraction 11, suggesting that complex 3 results from addition of TFIIB to complex 2 (TFIIA+TFIID). Addition of purified RNA pol II to a binding reaction containing TFIIA, -D, and -B (DEAE fraction 7) resulted in further formation of complexes 4 and 5 (Figure 5C, lane 1). Addition of only the DEAE gradient fractions 9–11 to this mixture generated complexes 6 and 7. These fractions contained the TFIIE transcription activity. It was not possible to test strict coelution of complex 3 formation with TFIIB activity, [illegible] over 6 and 7 formation with TFIIE activity as

[illegible] have interfered with the gel shift assay. The fact that complexes 6 and 7 are dependent on all the factors known to be required for in vitro transcription suggests that they represent complete initiation complexes. It is interesting to note that pol II-dependent complexes formed in pairs (complexes 4 and 5 and complexes 6 and 7). Possible reasons for this are discussed below.

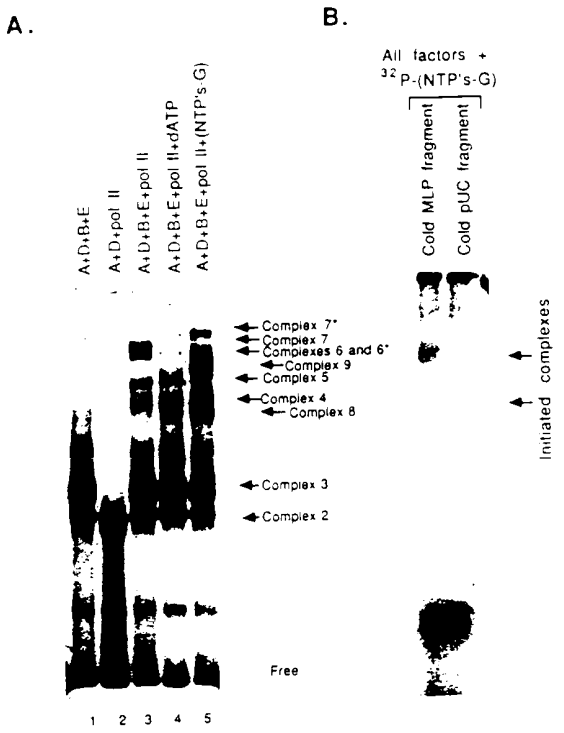

Figure 6. Effect of Nucleotide Triphosphates on the Transcription Factor–MLP Complexes

(A) Standard binding reactions were performed with the following protein components: 2.0 μl of calf thymus TFIIA, 2.0 μl of yeast TFIID, and 0.5 μl of HeLa fraction [CB] containing TFIIB and TFIIE (lane 1); 0.3 μl of calf thymus pol II added to TFIIA and TFIID (lane 2), or TFIIA, TFIID, fraction [CB], and pol II (lanes 3–5). In addition, the binding reaction in lane 4 contained 100 μM dATP. Besides the indicated protein components, the binding reaction in lane 5 also contained 60 μM ATP, 60 μM UTP, 60 μM CTP, and 10 μM 3′-O methyl GTP.

(B) Visualization of initiated transcription complexes by labeled RNA transcripts. Reactions contained the components described in part A, lane 5, except that the added CTP was 10 μM and labeled with ^{32}P on the α-phosphate (50 Ci/mmol). The DNA added was unlabeled. The reaction in lane 1 received 5 ng of a MLP fragment identical in sequence to the fragment used as probe in part A (containing MLP sequences −53 to +33), while the reaction in lane 2 received 5 ng of a similar sized fragment derived from pUC13. The reactions were incubated and electrophoresed exactly as the binding reactions in part A (see Experimental Procedures).

Effects of Nucleotide Triphosphates on Complexes

The effect of nucleotide triphosphates on the MLP-specific complexes was tested by addition of various combinations of nucleotides to the complete binding reaction. Initi- [illegible] has been shown to have an appro- [illegible] ATP (Bunick et al., 1982; Sawadogo and Roeder, 1984). Addition of ATP (data not shown) or dATP (Figure 6A, lane 4) caused two changes. The first was an apparent decrease in the mobility of complex 7. This same mobility shift was observed upon addition of either GTP or a non-hydrolyzable ATP analog (ADPNP), but not CTP or UTP (data not shown). It was also not observed when the potentially initiating dinucleotide ApC was added. Therefore, the mobility shift requires a purine nucleotide triphosphate, but apparently does not require γ-bond hydrolysis. Because of the broader nucleotide specificity, this effect is thought to be distinct from the energy requirement for transcription activation. The second change observed upon addition of ATP or dATP, but not any of the other nucleotides tested, was a substantial reduction in the amount of complexes 6 and 7 (and formation of complexes 6* and 7*, see below). With addition of dATP, a concommitent increase in the amount of complexes migrating at the rate of complexes 4 and 5 was usually observed. This is not apparent in Figure 6A, but has been observed in multiple experiments. Upon addition of ATP, but not dATP, complexes 4 and 5 also exhibited some reduction (data not shown). The difference between the effects of ATP and dATP may have to do with the additional role of ATP as the initiating nucleotide of the MLP transcript. The reduced amounts of these complexes in the presence of ATP were not due to a decrease in rate of formation, since preincubation of the binding components without ATP, followed by a short incubation with the nucleotide, also resulted in a comparable reduction (data not shown).

Because the first G residue of the MLP transcript is at position +11, addition of ATP, UTP, CTP, and 3′ O-methyl GTP (a chain terminator) yields transcription only to +11. Such a "paused" complex, assembled in crude extract (Cai and Luse, 1987a) or with partially purified factors (Sawadogo and Roeder, 1984), has been shown to be relatively stable. When assayed in the gel shift system, addition of these nucleotides to the reaction resulted in the formation of two new diffuse complexes: 8 and 9 (lane 5). Complex 8 migrated slightly faster than complex 4, and complex 9 migrated slightly slower than complex 5. When unlabeled MLP DNA was added in place of probe DNA to the transcription–binding reaction along with [α^{32}P]-CTP and ATP, UTP, and 3′ O-methyl GTP, label was incorporated in two diffuse complexes that migrated identically with complexes 8 and 9 (Figure 6B, lane 1). Such incorporation was not observed when a control DNA fragment of similar size was substituted for the MLP fragment (lane 2). Furthermore, labeled RNA recovered from these complexes migrated in a denaturing gel at the rates expected for accurately initiated MLP transcripts terminated at the first two G residues (data not shown). Formation of complexes 8 and 9, whether visualized by labeled DNA or RNA, was inhibited by the presence of 2 μg/ml of α-amanitin (data not shown). Therefore, complexes 8 and 9 probably represent pol II transcription complexes that have accurately initiated at the MLP.

[illegible] various complexes were determined by footprint analysis. For this, the complete binding mix was treated with DNAase I before loading onto the native gel. The DNA in each of the complexes was recovered and electrophoresed on a sequencing gel. The patterns of protection on the coding and noncoding strands are shown in Figure 7A and 7B, respectively. The free DNA (F) sample yielded

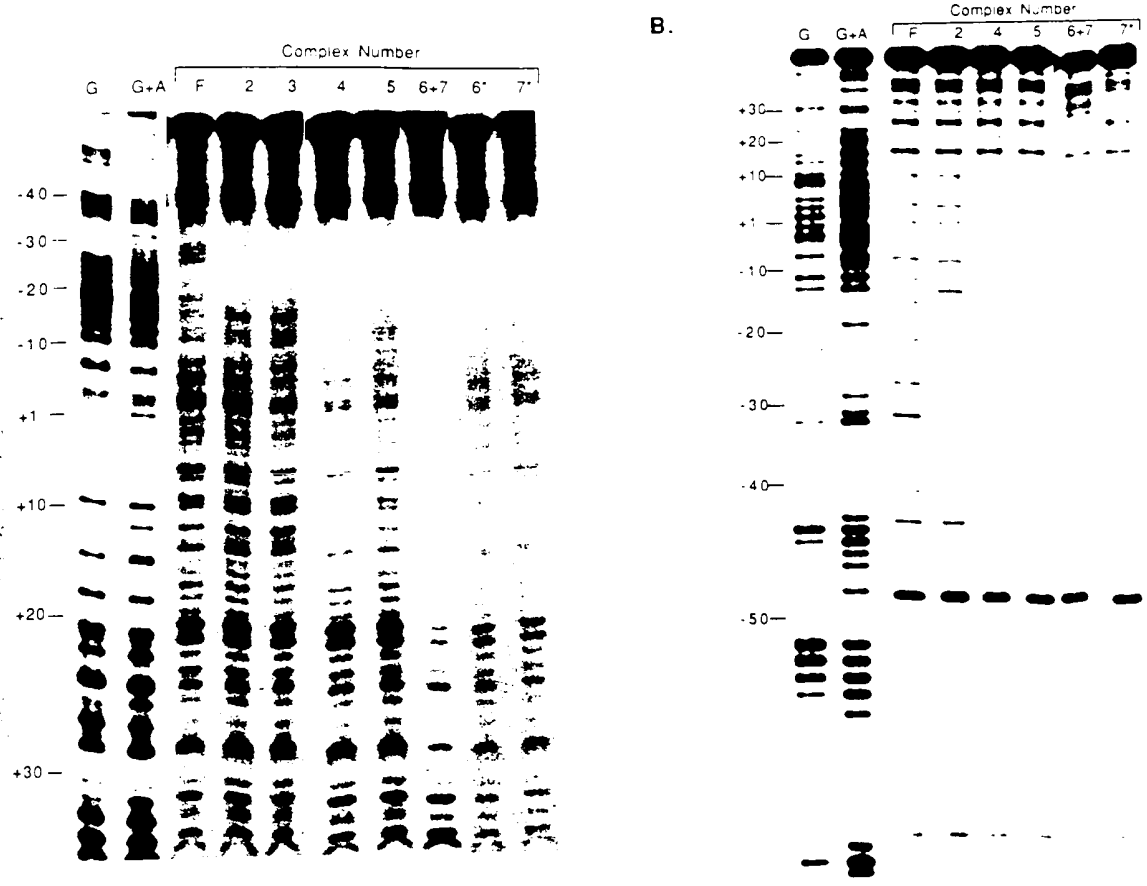

Figure 7. DNAase I Protection Patterns of MLP Sequences in the Transcription Factor–MLP Complexes

(A) Protection patterns on the coding strand. A binding reaction was performed as in Figure 6A, lane 3, except that all components were scaled up 5-fold. Probe DNA contained MLP sequences −53 to +33 and was 3′ end-labeled on the coding strand. The binding reaction was treated with DNAase I, electrophoresed on a native gel, and complex DNA was recovered as described in Experimental Procedures. Free DNA (F) or DNA recovered from each of the complexes was electrophoresed on a sequencing gel. DNAs from complexes 6 and 7 were not separated in this experiment. Complexes 6* and 7* were isolated by an identical protocol except that 100 μM dATP was added to the reaction. Numbers indicate nucleotide position relative to the transcription initiation site (+1). G and G+A are sequencing ladders of the same probe fragment.

(B) Protection patterns of the noncoding (template) strand. Experiments were done exactly as in (A), except that the probe DNA was labeled on the opposite strand. The protection pattern of complex 3 (data not shown) was determined in other experiments to be identical to that of complex 2. Similarly, the pattern of complex 6* was identical to that of complex 7*. Complexes 6 and 7 were separated in other experiments not shown, and always showed the same patterns on both strands. The low level of cleavage observed in the protected regions during analysis of complexes probably represents contributions of DNA from faster migrating species present as background in the native gel.

the same DNAase I pattern as naked DNA digested in solution. Complex 2 gave the same protection of the TATA element as was previously observed when TFIIA and TFIID were present in solution: approximately 25 bp spanning from −42 to −17.

Complex 3, which was only formed when TFIIB was added with TFIIA and TFIID, showed the same protection of th[illegible] addition[illegible] −10[illegible] noncoding strand, the complex 3 pattern yielded a pattern identical to that of complex 2 (data not shown). Therefore, TFIIB is probably bound in the complex downstream of the TATA element, perhaps loosely associated with regions of the coding strand. An assymetric association would be very interesting as it would leave the initiation site at the template (noncoding) strand accessible to the polymerase.

Complexes 4 and 5, which are generated when purified pol II is added to TFIIA, -IID, and -IIB, showed identical footprints in all cases. The coding strand had the same upstream boundary (−42) as complexes 2 and 3, but cleavages at sites downstream were strongly suppressed [illegible] The expanded protection relative to complex 3 presumably reflects the binding of the large RNA pol II molecule to the protein–DNA complex. This binding extends not only over the TATA element and the initiation site, but also two helical turns downstream of the initiation site. Complexes 6 and 7 were only observed when TFIIE was

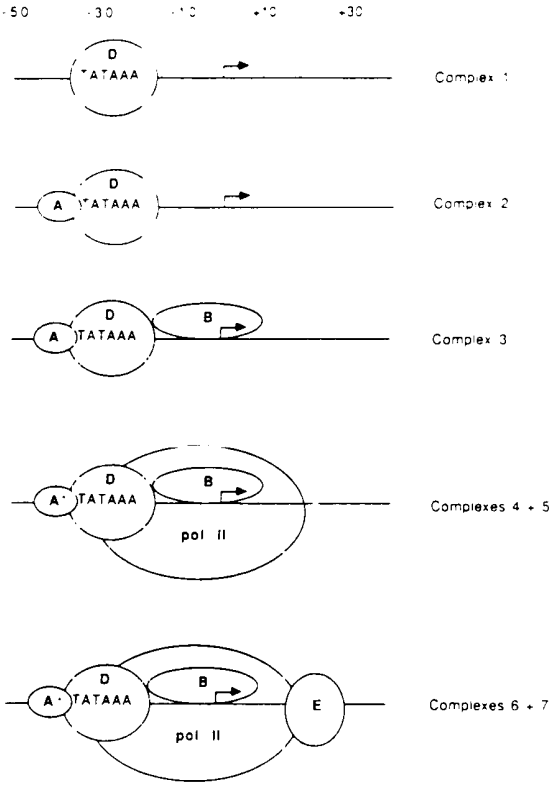

Figure 8. Schematic Model of the Molecular Structure of the Complexes

The factor requirements and DNAase I footprint patterns of each of the complexes are reflected in this diagram. The arrow indicates the transcription initiation site, and the numbers at the top are nucleotide positions relative to that site. The asterisk on TFIIA in complexes 4–7 is to signify that it was not possible to determine whether TFIIA was present in these complexes. Complexes 6 and 7 are believed to represent complete initiation complexes.

added to the components of complexes 4 and 5. The DNAase I protection patterns of complexes 6 and 7 were identical. The patterns were similar to those of complexes 4 and 5, but showed approximately 10 bp (one helical turn) extension of protection downstream. The coding strand was now protected to about +30, and the noncoding strand was protected to about +25 with some enhanced cleavage in sequences further downstream. These additional protections suggest that TFIIE binds downstream of the polymerase.

Addition of nucleotide triphosphates had no effect on [illegible] reaction caused not only a partial loss of the TFIIE-dependent protections between +20 and +30, but also a general suppression of all cleavage sites from +30 to the downstream end of the probe. As previously noted, complexes 6 and 7 apparently associate upon addition of ATP [illegible] ATP. These are the same nucleotide triphosphates that generate complexes 6* and 7*, which exhibit the downstream alterations in the footprint. Since the sets of complexes differ in the DNAase I pattern in an area of protection likely to be due to the TFIIE binding, it is theorized than an ATP-dependent release of TFIIE occurs during conversion of the complexes. This transition is probably distinct from the apparent mobility decrease observed upon conversion of complex 7 to 7*, because complex 6 undergoes the same footprint changes without an apparent mobility shift. In addition, some purine nucleotide triphosphates (such as GTP or ADPNP) will convert complex 7 to the slower migrating form without producing the changes in protection patterns (data not shown).

Addition of NTPs that allow transcription to +11 generated complexes 8 and 9. The protection patterns of complexes 8 and 9 were identical to each other and to those of complexes 4 and 5 (data not shown). However, since complexes 8 and 9 migrated with mobilities similar to complexes 4 and 5 respectively, it was difficult to prepare DNA confidently from the individual complexes. Therefore, the protection patterns of complexes 8 and 9 remain uncertain.

Discussion

We have resolved a series of complexes by native gel electrophoresis that suggest an ordered assembly of the general transcription factors and pol II on the Ad2 MLP template. The mobility of each complex, the factor requirements for its formation, and its DNAase I footprint have been combined to predict a molecular structure for each complex. A schematic representation of the composition and relative positions of the general transcription factors in each of these ordered complexes is shown in Figure 8. We propose that these complexes represent intermediates in the initiation reaction.

Complex 1

The first step in initiation is probably recognition of the TATA element by TFIID, perhaps in combination with TFIIA (see below). TFIID was required for formation of all of the specific complexes (2–7). TFIID will specifically protect the TATA element sequences −37 to −17 from DNAase I in the absence of any other transcription factors. A specific complex can be inferred from this result, and has been termed complex 1. For unknown reasons, no specific TFIID–TATA element complex was detectable in the gel shift assay. Complex 1 may be unstable in the gel shift assay, even though solution studies suggest a relatively long half-life (>20 min) for the TFIID–TATA element complex [illegible] from those observed with the yeast TFIID. The HeLa TFIID fractions were found to protect sequences from −38 to −4, followed by alternating hypersensitive sites (spaced about 10 bp apart) and protected areas to +35 (Sawadogo and Roeder, 1985b; Nakajima et al., 1988). While the protection in the region of the TATA element is similar to that seen with the purified yeast TFIID, the

downstream protections are strikingly different. Curiously, the downstream protections were observed on the MLP and the histone H4 promoter, but not several other promoters; these others showed only the TATA element protection (Nakajima et al., 1988). While the different TFIID footprints may reflect differences caused by proteolysis or conformation changes, another possible explanation is that the fractions containing mammalian TFIID (purified about 300-fold; Nakajima et al., 1988) also contain one or more other factors responsible for the downstream protections. Alternatively, it is possible that the yeast and mammalian factors are fundamentally different. These two factors have significantly different sizes when analyzed by sedimentation and chromatography (see Buratowski et al., 1988, for discussion).

Complex 2

Complex 2 contains both TFIID and TFIIA bound specifically to the −42 to −17 region of the MLP. The strongest evidence for the presence of TFIIA in the complex is that different sources of TFIIA transcription activity (calf thymus, HeLa cells, or yeast) generated complexes of different mobilities, but identical DNAase I footprints, in conjunction with TFIID. The heteromeric complex 2 is reminiscent of some upstream transcription factors. The yeast transcription activator HAP 2/3 (Hahn and Guarente, 1988) and a family of human CCAAT binding proteins (Chodosh et al., 1988a) require at least two different polypeptides to reconstitute sequence-specific binding in a gel shift assay. In addition, the heteromeric contacts between the subunits of the yeast and mammalian activators are highly conserved, as subunits from the different species can be combined to regenerate specific binding (Chodosh et al., 1988b). Similarly, the TFIID–TFIIA interactions must be conserved. Both mammalian and yeast TFIIA can combine with yeast TFIID to generate TATA element-specific complexes, and any combination of yeast and/or mammalian TFIIA and TFIID can function together in a reconstituted in vitro transcription reaction (data not shown).

Addition of TFIIA slightly enhanced the binding of TFIID and modified the DNAase I protection pattern of TFIID on the TATA element. Interestingly, TFIIA more effectively enhanced the binding of TFIID to a cryptic TATA sequence at −70 than to the MLP TATA element. Thus, TATA elements from different promoters may differ in their dependence upon TFIIA. Comparision of the footprints of complexes 1 and 2 demonstrates a TFIIA-dependent extension and a 3-fold enhancement in cleavage of a site at the upstream boundary of protection (about −43). Solely for this reason, TFIIA is positioned on the upstream side of TFIID in Figure 8.

com[illegible] and which was resistant [illegible] challenges with either [illegible] TATA element, poly (dI-dC), or Sarkosyl (Davison et al., 1983; Fire et al., 1984; Reinberg et al., 1987). The complex inferred from these solution studies probably corresponds to complex 2, as it has the same factor requirement and response to poly(dI-dC) challenge (data not shown). Studies [illegible] TFIIA in initiation have been [illegible]founded by a variable requirement for the factor in in vitro transcription reactions; reports have ranged from TFIIA being totally dispensable (Sawadogo and Roeder, 1985a) to being strongly stimulatory (Egly et al., 1984; Samuels and Sharp, 1986; Buratowski et al., 1988) to being absolutely required (Reinberg et al., 1987).

Order of addition experiments have led to the suggestion that TFIIA acts before binding of TFIID, perhaps through a nonspecific interaction with the DNA (Reinberg et al., 1987). This would be surprising, as TFIIA does not bind to negatively charged columns. A potential complication in such experiments is the DNA-dependent inactivation of mammalian TFIID during preincubation in the absence of TFIIA (Fire et al., 1984). While the results presented here do not argue for or against the hypothesis that TFIIA acts before TFIID, they do demonstrate that TFIIA remains stably associated after binding of TFIID to the TATA element, at least during the early steps of initiation complex formation.

Complex 3

After binding to TFIID and TFIIA to the TATA element, TFIIB probably binds to the initiation complex. Fractions containing TFIIB are required for formation of complexes 3–9. The evidence that TFIIB is actually present in complex 3 is the difference in mobilities of complexes 2 and 3, which is dependent on fractions known to contain TFIIB activity, and the differences in DNAase I protection in complex 3, compared with complex 2. TFIIA remains in complex 3, as the mobility of this complex varies with different sources of TFIIA (data not shown).

The DNAase I protection pattern of complex 3 is quite interesting. In addition to protection of the TATA element as observed in complex 2, partial protection of some cutting sites from −10 to +10 on the coding strand was observed. No corresponding protections were detected on the noncoding strand. This pattern suggests that TFIIB may be associated specifically with one strand and extend from the TFIID–TFIIA complex to beyond the transcription initiation site. Purified RNA pol II added to the binding mixtures did not generate any new complexes unless TFIIB as well as TFIID was present, suggesting that in the absence of TFIIB, polymerase does not bind stably to TFIID. It is interesting that TFIIB has been shown to associate with RNA pol II in solution (Zheng et al., 1987). Therefore, it seems likely that TFIIB acts as a "bridging" molecule between the TFIIA–TFIID–TATA element complex and pol II.

If TFIIB does act as a bridge, the TFIIB–pol II interaction may also be involved in "measuring" the distance from the TATA element to the initiation site. In this regard, it will be informative to isolate the yeast homolog of TFIIB, because [illegible] the domain of the TFIIB protein that involved in contacting the TFIIA.TFIID complex, must be conserved over evolution, since the yeast TFIID and TFIIA function with the other mammalian transcription factors. It remains to be seen whether the domain of TFIIB that interacts with polymerase is also conserved. The absence of this conserva-

tion may explain why we have so far been unable to substitute yeast pol II for the mammalian enzyme.

Complexes 4 and 5

The addition of purified RNA pol II is necessary for generation of complexes 4–7. As discussed above, polymerase does not stably bind to the TFIID–TATA element complex unless TFIIB is also present in the complex. Complexes 4 and 5 apparently represent binding of pol II to complex 3. Protection from DNAase I cleavage is quite extensive in these complexes, extending as far downstream as +20. In addition, the upstream boundary of protection on the template strand, which is about −43 in complex 3, extends to −47 upon pol II binding (complexes 4–7). If this protection is due to the polymerase molecule, and not a conformational change in TFIIA or TFIID, then a portion of pol II would be in close proximity to upstream sites and any stimulatory transcription factors bound there. The MLP transcription factor (MLTF or USF) binding site, which was not contained on the probes used in the gel shift assays presented here, is located from −50 to −66 (Carthew et al., 1985; Sawadogo and Roeder, 1985b; Moncollin et al., 1986). This juxtaposition would allow for direct interactions between polymerase and at least the most proximal upstream factor.

Curiously, the pol II-dependent complexes occur in pairs: complexes 4 and 5 (pol II binding without TFIIE) and complexes 6 and 7 (binding with TFIIE). The members of each pair always show identical DNAase I footprints. While the basis of the mobility difference between the members of each pair is not known, at least three plausible explanations exist. First, the mobility difference may be due to binding by different forms of pol II. It is well documented that purification of pol II results in the isolation of intact and proteolyzed forms (Hodo and Blatti, 1977). A second possibility is that the mobility difference reflects the binding of another protein to the RNA polymerase complex. A potential candidate is the protein S-II (also called TFIIS and RAP 38), a 37–40 kd molecule that has been shown to bind RNA pol II and to stimulate transcription elongation (Horikoshi et al., 1984; Reinberg and Roeder, 1987b; Sopta et al., 1985). A third possibility is that the mobility difference reflects a conformation change in the polymerase–DNA interaction. Such an explanation has been proposed for a doublet observed with purified E. coli polymerase. In similar gel assays, purified bacterial holoenzyme generated two promoter-specific complexes, the relative amounts of which could be affected by varying binding and gel temperatures (Straney and Crothers, 1985). Experiments [illegible]

[illegible] might be interpreted as indicating that TFIIA is not required for later steps in initiation complex formation. Interactions between TFIIB and TFIID may occur in the absence of TFIIA, since incubation of the purified TFIID and the TFIIB fraction generated a complex with a mobility intermediate to those of complexes 3 and 3 (data not shown and Figure 4). If TFIIA facilitates but is not absolutely required for complex formation, varying conditions (such as the concentration of the other factors) might make the rate of the TFIIA-promoted step limiting, or not limiting, and therefore lead to conflicting observations. An alternative explanation that cannot yet be excluded is that other fractions are contaminated with TFIIA activity. Either possibility is consistent with the partial requirement for added TFIIA in the reconstituted in vitro transcription reaction.

Kinetic experiments, involving preincubation of transcription factors with a DNA template, followed by challenge with inhibitors or a second template, have suggested an association of pol II with complexes dependent upon TFIIA and TFIID, in the absence of TFIIB and TFIIE (Fire et al., 1984; Reinberg et al., 1987). A stable association of pol II with the TFIIA–TFIID–TATA element complex was not observed in this study, nor in other studies that assayed stable complex formation by DNAase I footprint analysis (Van Dyke et al., 1988) or exchange of drug-resistant and -sensitive pol II (Carthew et al., 1988). Thus, it is likely that the earlier kinetic studies either were flawed by the use of contaminated fractions, or were complicated by a nonspecific association of pol II with the template DNA during preincubation, or that the hypothetical association between pol II and the TFIID–TFIIA–TATA element complex is not sufficiently stable to be detected in the other assays.

Complexes 6 and 7

Addition of TFIIE to complexes 4 and 5 generates complexes 6 and 7. TFIIE apparently binds downstream of the polymerase, protecting sequences in the +20 to +30 regions from DNAase I cleavage. TFIIE has been shown to bind pol II in solution (Reinberg and Roeder, 1987a). Indeed, TFIIE is related or identical to the RAP 30/74 complex (Flores et al., 1988), two complexed proteins that were isolated on the basis of their affinity for pol II immobilized on a column matrix (Sopta et al., 1985). The RAP 30/74 complex has been shown to be necessary for accurate transcription initiation (Burton et al., 1986), but can be substituted for by a partially purified TFIIE fraction (Flores et al., 1988). Although it is possible to separate TFIIB, TFIIE, and pol II and to construct complexes representing the binding of each factor, it is possible that in vivo pol II and TFIIE, and perhaps TFIIB, are associated before binding to the TFIID–TFIIA–TATA element complex.

Fractions containing TFIIE (Sawadogo and Roeder, 1984; Reinberg and Roeder, 1987a) and RAP 30/74 (Burton et al., 1986) have been reported to contain a DNA-dependent ATPase activity. Transcription has an energy [illegible]

[illegible] and Roeder, 1984). These same [illegible] nucleotides cause an apparent dissociation of complexes 6 and 7 and generation of complexes 6* and 7*. At least with dATP, the loss of complexes 6 and 7 also correlated with an apparent increase in complexes with mobility similar to complexes 4 and 5. This dissociation is accompanied by a loss of [illegible] complexes 6* and 7* of the TFIIE-dependent DNAase protection between +20 and +30. A similar loss of

DNAase I protection was observed following nucleotide triphosphate addition to transcription complexes assembled in solution on the MLP in nuclear extracts (Cai and Luse, 1987b) or with partially purified factors (Van Dyke et al., 1988). Both studies showed that complete complexes had an upstream DNAase I protection boundary of about −42, and a downstream boundary of +30 that retreated to +25 upon addition of either ATP or dATP. Although these studies did not have the sensitivity of the native gel shift assay to resolve the intermediate complexes 3–5, the protections described were very similar to the protection seen with complexes 6 and 7, and probably represent the same entity: a complete initiation complex. We propose that the loss of the downstream protection and the instability of complexes 6 and 7 result from an ATP/dATP-dependent dissociation of TFIIE from the initiation complex. This may be related to the DNA-dependent ATPase detected in TFIIE fractions. One likely function for a DNA-dependent ATPase that binds downstream of the polymerase molecule might be as a helicase, which would unwind the DNA, making the template strand accessible to the polymerase. On linear molecules such as those used in this study, the putative helicase would run off the template, which would explain the ATP-dependent dissociation of TFIIE.

Complexes 8 and 9

Addition of NTPs that allow transcript elongation to +11 results in formation of complexes 8 and 9. These complexes have been shown to contain accurately initiated transcripts. Complexes 8 and 9 migrate near complexes 4 and 5 respectively, consistent with the model that TFIIE dissociates from the activated transcription complex. The toxin α-amanitin blocks formation of complexes 8 and 9, but not other complexes, and not the ATP/dATP effects discussed above. This is consistent with its role as an inhibitor of elongation, but not binding, by pol II (Cochet-Meilhac and Chambon, 1974).

The identification of an ordered set of complexes, each containing some subset of the previously isolated general transcription factors, defines a reaction pathway for transcription initiation by pol II. While the binding of each factor to the complex may not be strictly sequential in vivo, with some binding together as pre-existing complexes, it is likely that the contacts between factors defined by the ordered pathway shown in Figure 8 occur in vivo. As has been long appreciated, initiation of transcription in eukaryotic systems is a complicated reaction, involving many proteins. As a conceptual analogy, prokaryotic DNA replication seems to be more applicable than prokaryotic

[illegible] it appear that analysis of complexes by native gel electrophoresis will not only be useful for further elucidating the mechanisms of action of the general factors in transcription, but also for discovering which step(s) and general factors are affected by the regulatory transcription factors that bind upstream of the TATA element.

Experimental Procedures

Protein Purification

RNA polymerase II was purified from calf thymus according to Hodo and Blatti (1977) to a final protein concentration of 150 μg/ml. By silver-stained gel analysis, the polymerase is ~90% pure and has the expected subunit composition. TFIID was purified approximately 90,000-fold from S. cerevisiae, to the Superose-12 FPLC step, as previously described (Buratowski et al., 1988). The final protein concentration was 2–5 μg/ml, and the protein was judged to be 30–50% pure by silver-stained gel analysis (Hahn et al., unpublished data). Calf thymus TFIIA was purified to step V (9,000-fold; 2.3 μg/ml of final protein concentration) as previously described (Samuels and Sharp, 1986). HeLa fraction [AB] (Samuels et al., 1982), which had a protein concentration of 4 mg/ml, was used as a source of partially purified human TFIIA. Yeast TFIIA was partially purified by chromatography of a yeast extract over Heparin-Sepharose and DEAE-Sepharose to a final protein concentration of 0.5 mg/ml; the detailed purification will be published elsewhere (Hahn et al., unpublished data). HeLa fraction [CB] (Samuels et al., 1982) was used as the source of partially purified human TFIIB and TFIIE. Separation of TFIIB and TFIIE was carried out in two ways. One ml of fraction [CB] (7 mg of protein/ml) in Buffer A + 100 mM KCl (Samuels et al., 1982) was loaded onto a 1 ml DEAE-Sephacel column (Pharmacia) at a flow rate of 3 column volumes/hr. The flowthrough fraction (termed fraction [CBA], containing 1 mg of protein/ml) was collected and contained the majority of TFIIB activity (Dignam et al., 1983b). After washing with three column volumes of Buffer A + 100 mM KCl, the column was then eluted with three column volumes of Buffer A + 300 mM KCl. Fractions from this wash were pooled (fraction [CBB], 3.5 mg of protein/ml) and contained the majority of TFIIE activity. Alternatively, separation was carried out by gradient elution of the DEAE-Sephacel column. The loading procedure was the same, except that the sample was diluted so that the loading buffer was Buffer A + 50 mM KCl. The column was then eluted with a 5 column volume linear gradient of Buffer A from 50 to 500 mM KCl. Fractions of 300 μl were collected and assayed as described below. TFIIB activity was found to peak at 75 mM KCl, while TFIIE was found to elute at 200 mM KCl.

DNA Probes

DNA for probes was from the plasmids pRW (MLP sequences −53 to +33 cloned into the Sma site of pUC13) or pLP (−174 to +33; Chodosh et al., 1986). Fragments were prepared by excising the MLP inserts with either EcoRI and HincII or EcoRI and HindIII (NEB). Probes were made by end-labeling the EcoRI–HincII fragment with Klenow enzyme (Boehringer Mannheim) and [α^{32}P]-dATP(NEN) or, to label the other strand, by labeling the EcoRI–HindIII fragment with [α^{32}P]-dCTP-(NEN). The pUC fragment used as a negative control in the RNA labelling of initiated transcription complexes (Figure 6B) was a 140 bp PvuII–HindIII fragment excised from pUC13 that contained the polylinker. Probes and competitors were gel-purified.

Gel Electrophoresis DNA Binding Assay

The indicated protein components were incubated with 0.5–2 ng of probe (roughly 20,000 cpm) for 20–30 min at 30°C. All binding reactions were done at transcription buffer conditions (12 mM HEPES-NaOH[pH 7.9], 12% glycerol, 1 mM EDTA, 0.6 mM DTT, 60–100 mM KCl, 5 mM $MgCl_2$) and also contained 0.2 mg/ml of BSA and 5–20 μg/ml of poly(dG·dC):(dG·dC) in a total volume of 10 μl. Reactions were loaded onto 4–5% acrylamide native gels (0.15 × 16 cm, 40:1 mono. bis ratio, 2.5% glycerol) and run in buffer consisting of 25 mM Tris base, 190 mM glycine, and 1 mM EDTA (final pH 8.5). Electrophoresis was

[illegible]

DNAase I Footprinting

Binding reactions were performed as described above for the gel electrophoresis DNA binding assay. DNAase I (Worthington) was then added at approximately 10 μg/ml for 1 min at room temperature. For solution footprinting, the reactions were stopped with transcription stop mix (Samuels et al., 1982), phenol:chloroform extracted, chloroform extracted, EtOH precipitated, washed with 70% EtOH, dissolved in loading buffer (95% formamide, 1× TBE), heat denatured, and run

[illegible] gels immediately after DNAase I treatment. After native gel electrophoresis, the complexes were electroblotted to NA45 paper (Schleicher and Schull) in native gel running buffer (about 2 hr at 80 mA in a Bio-Rad Transblot electroblotting apparatus). The NA45 membrane was then autoradiographed, and the areas corresponding to the labeled complexes were cut out. Labeled DNA was eluted from the membrane into buffer containing 10 mM Tris–HCl (pH 7.9), 1 mM EDTA, and 1 M NaCl at 68°C for 20 min. The supernatant was then extracted, processed, and electrophoresed as described for solution footprinting.

In Vitro Transcription

Transcriptions were performed essentially as previously described (Buratowski et al., 1988). Assays for TFIIB and TFIIE together contained 0.5 μl (0.34 μg) of yeast TFIID (Mono S fraction), 0.5 μl of HeLa fraction [AB], which contains TFIIA, 0.5 μl of HeLa fraction [CD], and 0.2 μl of calf thymus RNA pol II in addition to the standard buffer, template, and nucleotide concentrations. Assays for TFIIE in addition contained 0.5 μl of the DE-B-Sepharose flowthrough fraction (DEB) described above, as a source of TFIIB. Assays for TFIIB [illegible] additional 1 μl of the 300 mM KCl fraction (DBE) of the DE-B-Sepharose column, as a source of TFIIE.

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References

Allison, L. A., Moyle, M., Shales, M., and Ingles, C. J. (1985). Extensive homology among the largest subunits of eukaryotic and prokaryotic RNA polymerases. Cell *42*, 599–610.

Bunick, D., Zandomeni, R., Ackerman, S., and Weinmann, R. (1982). Mechanism of RNA polymerase II–specific initiation of transcription in vitro: ATP requirement and uncapped runoff transcripts. Cell *29*, 877–886.

Buratowski, S., Hahn, S., Sharp, P. A., and Guarente, L. (1988). Function of a yeast TATA element-binding protein in a mammalian transcription system. Nature *334*, 37–42.

Burton, Z. F., Ortolan, L. G., and Greenblatt, J. (1986). Proteins that bind to RNA polymerase II are required for accurate initiation of transcription at the adenovirus 2 major late promoter. EMBO J. *5*, 2923–2930.

Burton, Z. F., Killeen, M., Sopta, M., Ortolan, L. G., and Greenblatt, J. (1988). RAP 30/74: a general initiation factor that binds to RNA polymerase II. Mol. Cell. Biol. *8*, 1602–1613.

Cai, H., and Luse, D. S. (1987a). Transcription initiation by RNA polymerase II in vitro: properties of preinitiation, initiation and elongation complexes. J. Biol. Chem. *262*, 298–304.

[illegible] polymerase [illegible] transcription factor binds to an upstream [illegible] adenovirus major late promoter. Cell *43*, 439–448.

Carthew, R. W., Samuels, M., and Sharp, P. A. (1988). Formation of transcription preinitiation complexes with an amanitin-resistant RNA polymerase II. J. Biol. Chem. *263*, 17128–17135.

[illegible] Sentenac, A., Egly, [illegible]

[illegible] Chodosh, L. A., Carthew, R. W., and Sharp, P. A. (1986). [illegible] peptide possesses the binding and transcription activities of the adenovirus major late promoter factor. Mol. Cell. Biol. *6*, 4723–4733.

Chodosh, L. A., Baldwin, A. S., Carthew, R. W., and Sharp, P. A. (1988a). Human CCAAT-binding proteins have heterologous subunits. Cell *53*, 11–24.

Chodosh, L. A., Olesen, J., Hahn, S., Baldwin, A. S., Guarente, L., and Sharp, P. A. (1988b). A yeast and a human CCAAT-binding protein have heterologous subunits that are functionally interchangeable. Cell *53*, 25–35.

Cochet-Meilhac, M., and Chambon, P. (1974). Mechanisms of the inhibition of RNA polymerases B by amatoxins. Biochim. Biophys. Acta *353*, 160–184.

Corden, J. L., Cadena, D. L., Ahearn, J. M., and Dahmus, M. E. (1985). A unique structure at the carboxyl terminus of the largest subunit of eukaryotic RNA polymerase II. Proc. Natl. Acad. Sci. USA *82*, 7934–7938.

Davison, B. L., Egly, J., Mulvihill, [illegible] mation of stable preinitiation complexes between eukaryotic class B transcription factors and promoter sequences. Nature *301*, 680–686.

Dignam, J. D., Lebowitz, R. M., and Roeder, R. G. (1983a). Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucl. Acids Res. *11*, 1475–1489.

Dignam, J. D., Martin, P. L., Shastry, B. S., and Roeder, R. G. (1983b). Eukaryotic gene transcription with purified components. Meth. Enzymol. *104*, 582–598.

Egly, J. M., Miyamoto, N. G., Moncollin, V., and Chambon, P. (1984). Is actin a transcription initiation factor for RNA polymerase B? EMBO J. *3*, 2363–2371.

Fire, A., Samuels, M., and Sharp, P. A. (1984). Interactions between RNA polymerase II, factors, and template leading to accurate transcription. J. Biol. Chem. *259*, 2509–2516.

Flores, O., Maldonado, E., Burton, Z., Greenblatt, J., and Reinberg, D. (1988). Factors involved in specific transcription by mammalian RNA polymerase II: RNA polymerase II-associating protein 30 is an essential component of transcription factor IIF. J. Biol. Chem. *263*, 10812–10916.

Guarente, L. (1987). Regulatory proteins in yeast. Annu. Rev. Genet. *21*, 425–452.

Guarente, L. (1988). UASs and enhancers: common mechanism of transcriptional activation in yeast and mammals. Cell *52*, 303–305.

Hahn, S., and Guarente, L. (1988). Yeast HAP2 and HAP3: transcriptional activators in a heteromeric complex. Science *240*, 317–321.

Hodo, H. G., and Blatti, S. P. (1977). Purification using polyethylenimine precipitation and low molecular weight subunit analysis of calf thymus and wheat germ DNA-dependent RNA polymerase II. Biochem. *16*, 2334–2343.

Horikoshi, M., Sekimizu, K., and Natori, S. (1984). Analysis of the stimulatory factor of RNA polymerase II in the initiation and elongation complex. J. Biol. Chem. *259*, 608–611.

Manley, J., Fire, A., Cano, A., Sharp, P. A., and Gefter, M. (1980). DNA-dependent transcription of adenovirus genes in a soluble whole-cell extract. Proc. Natl. Acad. Sci. USA *77*, 3855–3859.

Matsui, T., Segall, J., Weil, P. A., and Roeder, R. G. (1980). Multiple factors required for accurate initiation of transcription by purified RNA [illegible] B. Chem. *255*, 11992–11996.

[illegible] Nakatani, Y., Horikoshi, M., and Roeder, R. G. (1988). Factors involved in specific transcription by mammalian RNA polymerase. I. Purification, genetic specificity, and TATA box-promoter interactions of TFIID. Mol. Cell. Biol. *8*, 4028–4040.

Reinberg, D., and Roeder, R. G. (1987a). Factors involved in specific transcription by mammalian RNA polymerase. [illegible] purification and func-

tional analysis of initiation factors IID and IIE. J. Biol. Chem. 262, 3310–3321.

Reinberg, D., and Roeder, R. G. (1987b). Factors involved in specific transcription in mammalian RNA polymerase II. Transcription factor IIS stimulates elongation of RNA chains. J. Biol. Chem. 262, 3331–3337.

Reinberg, D., Horikoshi, M., and Roeder, R. G. (1987). Factors involved in specific transcription in mammalian RNA polymerase II: Functional analysis of initiation factors IIA and IID, and identification of a new factor operating at sequences downstream of the initiation site. J. Biol. Chem. 262, 3322–3330.

Samuels, M., and Sharp, P. A. (1986). Purification and characterization of a specific RNA polymerase II transcription factor. J. Biol. Chem. 261, 2003–2013.

Samuels, M., Fire, A., and Sharp, P. A. (1982). Separation and characterization of factors mediating accurate transcription by RNA polymerase II. J. Biol. Chem. 257, 14419–14427.

Sawadogo, M., and Roeder, R. G. (1984). Energy requirement for specific transcription initiation by the human RNA polymerase II system. J. Biol. Chem. 259, 5321–5326.

Sawadogo, M., and Roeder, R. G. (1985a). Factors involved in specific transcription by human RNA polymerase II: Analysis by a rapid and quantitative in vitro assay. Proc. Natl. Acad. Sci. USA 82, 4394–4398.

Sawadogo, M., and Roeder, R. G. (1985b). Interaction of a gene-specific transcription factor with the adenovirus major late promoter upstream of the TATA box region. Cell 43, 165–175.

Sopta, M., Carthew, R. W., and Greenblatt, J. (1985). Isolation of three proteins that bind to mammalian RNA polymerase II. J. Biol. Chem. 260, 10353–10360.

Straney, D. C., and Crothers, D. M. (1985). Intermediates in transcription initiation from the *E. coli lac* UV5 promoters. Cell 43, 449–459.

Struhl, K. (1987). Promoters, activator proteins, and the mechanism of transcriptional initiation in yeast. Cell 49, 295–297.

Van Dyke, M. W., Roeder, R. G., and Sawadogo, M. (1988). Physical analysis of transcription preinitiation complex assembly on a class II gene promoter. Science 241, 1335–1338.

Weil, P. A., Luse, D. S., Segall, J., and Roeder, R. G. (1979). Selective and accurate initiation of transcription at the ad2 major late promoter in a soluble system dependent on purified RNA polymerase II and DNA. Cell 18, 469–484.

Zheng, X.-M., Moncollin, V., Egly, J.-M., and Chambon, P. (1987). A general transcription factor forms a stable complex with RNA polymerase B (II). Cell 50, 361–368.